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Complete genomic characterization and antigenic relatedness of genogroup III, genotype 2 bovine noroviruses

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Summary. Bovine enteric noroviruses form a genogroup, III, distinct from the 2 human norovirus genogroups, I and II. Two genogroup III genotypes were suggested by partial genomic analyses. In the present study, analysis of the full-length genome sequence of Bo/Newbury2/76/UK and the more contemporary Newbury2-like virus, Bo/Dumfries/1994/UK, showed that both were 7311 nucleotides in length and had three open reading frames (ORFs), amino acids motifs typical of noroviruses, and 95% or greater amino acid identities to each other in all regions of their genome. Apart from the ORF1 NTPase region, their ORF1 regions had less than 90% identity to the genogroup III genotype 1 Bo/Jena/80/DE virus, confirming two genogroup III genotypes. A close antigenic relationship was demonstrated by ELISA between the genotype 2 viruses, which will allow their serological diagnosis.

Introduction

Noroviruses are a well-studied, leading cause of non-bacterial gastroenteritis in human adults, classified in the family *Caliciviridae* [10]. Recently, bovine viruses identified some years ago were identified as noroviruses [5, 25]. Human noroviruses belong to genogroups I and II [10], whereas the bovine noroviruses have been placed in a separate genogroup, III [1, 5, 25, 33]. Comparisons of partial genomic sequences of the two original bovine viruses identified two genetic clusters (genotypes) within genogroup III, represented by the Bo/Jena/80/DE virus (genogroup III/1), and the Bo/Newbury2/76/UK virus (genogroup III/2).

*Deceased

The complete genome sequence of the Jena virus is available, but the complete genome sequence of Newbury2, or any other bovine genogroup III/2 virus, has not been determined.

Noroviruses are non-enveloped particles with positive-sense, single-stranded, RNA genomes composed of 3 overlapping open reading frames (ORFs). Each encodes a single protein: ORF1, the polyprotein; ORF2, the major capsid structural protein; and ORF3, a minor structural protein [4, 8]. The polyprotein is proteolytically cleaved to produce the N-terminal, NTPase, VPg, 3C-protease and 3D-polymerase proteins. The same genome organization and conserved amino acid motifs, GPPGIGKT (in the NTPase), GDCG (in the 3C-protease), and GLPSG and YGDD (in the 3D-polymerase), have been found in the polyproteins of all the human noroviruses examined and the bovine norovirus Jena [7, 16, 20, 23, 25, 26]. However, Newbury2-like bovine noroviruses have not been fully characterized in the ORF1 polyprotein region.

In the last few years, RT-PCR using primers based on polymerase gene sequences has detected bovine Newbury2-like noroviruses from geographical locations throughout the world [30, 36, 44, 45]. Data based on polymerase and capsid sequence suggested that Newbury2-like viruses are closely related to each other and to the proposed prototype genogroup III/2 Newbury2 virus identified nearly 3 decades previously. Knowledge about the antigenic relatedness of bovine noroviruses is required for serological diagnoses and seroprevalence studies and if vaccination is proposed. The study of norovirus antigenicity was hampered by the inability to culture these viruses, but this has been circumvented by the use of recombinant virus-like particles (VLPs) for both human and bovine noroviruses in enzyme-linked immunoabsorbant assays (ELISAs) [6, 14, 17, 18, 29]. The antigenic relatedness of Newbury2-like viruses has not been studied as it has not been cultured or its capsid protein expressed as VLPs.

The present study examined, for the first time, the complete genome sequence and organization of a genogroup III/2 virus, Bo/Newbury2/1976/UK and a second, more contemporary genogroup III/2 Newbury2-like virus, Bo/Dumfries/1994/UK. Newbury2 VLPs were expressed from baculovirus and used in an ELISA to monitor antibody development in experimentally infected calves and to assess the antigenic relatedness of Bo/Newbury2/1976/UK and Bo/Dumfries/1994/UK.

Materials and methods

Viruses

Bo/Newbury2/1976/UK (previously named Newbury SRV-2) was identified in southern England in association with calf diarrhoea in 1976 [44]. Bo/Dumfries/1994/UK was identified in Dumfries, Scotland in 1994 by Dr. D. Snodgrass [33].

Experimental infection of calves and production of convalescent antisera

Four gnotobiotic calves (P131, P137, P142 and Q21) were orally inoculated with Newbury2 virus as part of a cross-protection experiment between the Newbury1 (previously named Newbury SRV-1) and Newbury2 viruses, as reported previously [3]. One calf (P131) served

as a homologous challenge control and was given a primary inoculation with Newbury2 plus primary and secondary challenge inoculations with Newbury2. Two calves (P137 and P142) were given a primary inoculation and primary challenge inoculation with Newbury2 and secondary challenge to Newbury1. The fourth calf (Q21) served as a Newbury2 challenge control and was inoculated for the first time with Newbury2 at the time of challenge. Pre-inoculation and post-primary, post-primary challenge and post-secondary challenge sera were taken (see Table 2) and had been stored at −20°C. No evidence of antibody degradation was obtained during storage by monitoring antibody titres from calves infected with bovine rotavirus (unpublished). Two gnotobiotic calves were orally inoculated with Bo/Dumfries/1994/UK, and sera were taken 14 days later, then again 8 and 15 days after homologous challenge. Bovine antisera from calves inoculated with bovine rotavirus UK, the bovine enteric coronavirus, bovine astrovirus and the Haden strain of parvovirus [3] were used in the Newbury2 ELISA to determine specificity.

Genome sequencing

Viral RNA extraction and reverse transcription were performed as described previously [30] using MMLV reverse transcriptase and random hexamers (Promega). PCR was performed using the Hi Fidelity PCR system (ROCHE). An amino acid alignment of polyproteins from the genus *Norovirus* (Bo/Jena/1980/DE, 17 human noroviruses from genogroup I or II plus the murine norovirus) was used to identify conserved sequences in the NTPase region of the polyprotein, DRIEK/NK and APQGGFD. Primers JENA01 and JENA02 were designed to the conserved region in the NTPase corresponding to the Jena virus nucleotide sequence (Table 3). The subsequent nucleotide sequence of a 262-bp amplicon generated from the Dumfries genome that was cloned into a pCR2.1-TOPO vector was used to design additional primers, which were used to amplify two regions (JANA01/BoCVhelR_02 – 1851 nucleotides; BoCVhel_01/BoCVproR_01 – 1944 nucleotides) of the Newbury2 and Dumfries genomes. Amplicons cloned into pCR2.1 (Invitrogen) were sequenced in both directions using overlapping primers (Table 3). The 5′ end of the Newbury2 genome was deduced using 5′ rapid amplification of cDNA ends (RACE). cDNA generated by reverse transcription using the primer BoCV5′RACE_05 was purified using a QIAquick gel extraction kit (Qiagen). Terminal deoxynucleotide transferase (Promega) was used to add a poly-C tail to the 5′end of the purified cDNA. The 5′end of the Newbury2 genome was amplified using the Hi Fidelity PCR system (ROCHE) with an abridged anchor primer (Invitrogen) and BoCV5′RACE_06 (Table 3). The resulting 337-bp amplicon was cloned into pCR2.1, and 5 clones were sequenced in both directions to establish a consensus nucleotide sequence. The MRC gene service performed sequencing and the sequences were assembled using Staden [39].

The Simplot computer program [27] was used to generate identity plots of the complete genomes and the complete ORF1 polyproteins of the bovine noroviruses Newbury2, Dumfries and Jena using a window size of 200 and step size of 20 with gap strip off and J-C correction on. A multiple alignment of the translated amino acid sequences of the complete polyprotein (ORF1) was generated using Clustal X version 1.8 [43], which was used for phylogenetic analyses. PHYLIP (J. Felsenstein, Department of Genetics, University of Washington, Seattle; Phylogeny Inference Package, version 3.5c) was used for parsimony (DNApars and Protpars), UPGMA, Fitch-Margoliash and bootstrap analyses, plus TreePuzzle 5.2 [37] for additional maximum likelihood with quartet puzzling analyses.

The nucleotide sequence accession numbers for the noroviruses referred to in the text are: Genogroup I; Norwalk – NC_001959, Southampton – L07418, Chiba – AB042808, WUG1 – AB081723, SzUG1 – AB039774: Genogroup II; Snow Mountain – AY134748, Lordsdale – X86557, Langen1061 – AY485642, Gifu – AB045603, U1 – AB039775, U3 – AB039776, U4 – AB039777, U16 – AB039778, U17 – AB039779, U25 – AB039780, U18 – AB039781,

U201 – AB039782: Genogroup III; Jena – AJ011099, Newbury2 – AF097917, Dumfries – AY126474: Unassigned; Murine – AY228235.

Expression of the Newbury2 capsid protein as VLPs

The primers BoCVCap5′*Eco*RI (5′-GAATTCATGAAGATGACTGACAGA-3′ – the initiation codon is underlined) and NA2Capsid3′*Xho*I (5′-CTCGAGTCAGAAGCCATGAAG GCG-3′ – the reverse complementary sequence of the termination codon is underlined) were designed using the genomic sequence at the 5′ and the 3′ end of the Newbury2 ORF2 gene that incorporated the restriction sites *Eco*RI and an *Xho*I. A 1581-bp RT-PCR amplicon of the expected size was cloned using pCR2.1-TOPO-TA cloning kit (Invitrogen) to create the construct pCR2.1-Newbury2-ORF2. A clone of pCR2.1-Newbury2-ORF2, which had been sequenced in both directions to confirm the integrity of ORF2 gene, and the pFastBac^{TM} 1 transfer vector (Invitrogen) were digested with *Eco*RI and *Xho*I (Promega). The Newbury2-ORF2 gene and the pFastBac^{TM} 1 transfer vector were ligated to create the construct pFastBac^{TM} 1-Newbury2-ORF2 that was used to transform TOP10 *E. coli* from which the construct was amplified and purified.

Donor plasmid pFastBac^{TM} 1-Newbury2-ORF2 was transformed into DH10Bac (Invitrogen) competent cells for transposition. After the recombinant bacmids were determined to be correct by two successive blue-white colonies analysis, the recombinant bacmid DNA was used to lipofect *Spodoptera frugiperda* (Sf9) cells. The resulting recombinant baculovirus was amplified twice, and expression of ORF2 was checked by Coomassie blue staining of SDS-PAGE of infected Sf9 cells lysate. For VLP production, Sf9 cell monolayers were infected with recombinant baculovirus at a multiplicity of infection of 5 PFU/cell and incubated in Hink's medium supplemented with 1% fetal calf serum for 5 days at 26 °C. VLPs were extracted from supernatant of freezed-thawed infected Sf9 cells as described previously [22] except that Freon 113 was substituted by Vertrel XF as described by Mendez et al. [28]. VLPs were purified by isopicnic centrifugation in cesium chloride gradients and quantified by the method of Bradford using bovine serum albumin as standard. Recombinant VLPs were negatively stained with 2% uranyl acetate and examined in a Philips CM12 electron microscope operated at 80kV. Micrographs were taken at 25,000 magnification on Kodak SO163 Image plates.

Western blot analysis

Bovine serum albumin (BSA) was used to establish the protein concentrations of double CsCl-purified VLPs with a Micro-BCA assay (Pierce). The Newbury2 capsid protein (250 ng/lane) was blotted onto nitrocellulose membranes (Invitrogen) following electrophoresis with PAGEr Duramide 4–20% Tris–Glycine pre-cast gels (Cambrex) and western transfer. Prior to immunostaining, the nitrocellulose membranes were stained with Ponceau S (Sigma) to establish that the Newbury2 capsid protein had been successfully transferred. Membranes were blocked with 2% non-fat dried skimmed milk in Tris-buffered saline (TBS; 10mM Tris–HCl [pH 7.4], 0.15 M NaCl) and washing steps performed with TBST (TBS + 0.05% Tween 20). Convalescent bovine antisera to Newbury2 and rotavirus UK (as a negative control serum) were diluted 1:100 in TBST + 1% non-fat dried skimmed milk powder and detected using a 1:5000 dilution of rabbit anti-bovine IgG horseradish-peroxidase conjugate (Sigma). HRP activity was detected using TMB (3,3′,5, 5′-tetramethylbenzidine) stabilised substrate (Promega). The molecular mass of the Newbury agent-2 capsid protein was calculated from a standard curve produced using the Prosieve Color (Cambrex) molecular mass marker.

Antibody detection ELISA

Maxisorb plates (Nunc) were coated overnight at 4 °C with 5 μg/ml of antigen in carbonate buffer pH 9.6. Antigens used were double CsCl-purified Newbury2 VLPs or rotavirus 2/6 VLPs produced from the bovine rotavirus RF strain, or the supernatant from wild-type baculovirus-infected Sf9 cells (mock antigen). Plates were blocked with 2% non-fat dried skimmed milk in PBS and washed with PBST (PBS + 0.05% Tween 20 [Sigma]) between steps. Antisera from experimental calves were serially diluted from 1:50 in a 2-fold dilution series in 1% non-fat dried skimmed milk in PBST, dispensed into wells previously coated with test or mock antigen, and incubated at room temperature for 1 h. Binding was detected by incubation with a 1:10,000 dilution of rabbit anti-bovine IgG horseradish peroxidase-conjugate (Sigma) in 1% non-fat dried skimmed milk in PBST at room temperature. TMB was used as substrate (Sigma). Serum titres were calculated by linear regression using Prism 4 (GraphPad Software). Endpoints were defined as the highest dilution at which the net absorbance value (absorbance units of the serum with the test antigen minus absorbance units with the mock antigen) was twice the absorbance value for antigen-coated wells without a test bovine serum.

Molecular modelling

The homology model of the Newbury2 capsid protein was generated using the translated amino acid sequence of the complete Newbury2 ORF2 gene, which was submitted to the Swiss-model server http://swissmodel.expasy.org/. The software programme VMD 1.8.2 [15] was used to visualise the co-ordinate data and generate the image, which was rendered using Pov-ray™ for Windows™.

Results

Analysis of the complete Bo/Newbury2/1976/UK genome and ORF1 gene

Completion of the sequence of the Newbury2 genome showed that it was 7311 nucleotides in length, excluding the poly-A tail, that is, 27 nucleotides shorter than the Jena virus genome. The shorter length was attributed to deletions in the Newbury2 ORF3 gene and the shorter 3′ untranslated region reported previously [33]. The 5′ untranslated region of Newbury2 was identical in length (21 nucleotides) and composition to that of Jena [25] except for a single transition substitution (U–C) at the 21st nucleotide. The G + C content of the Newbury2 genome was 57%, similar to that of Jena (56%) [25].

The Newbury2 ORF1 gene was 5055 nucleotides in length and coded for a protein of 1684 amino acids with a predicted molecular mass of 186 kDa. It was 12 nucleotides longer than the Jena ORF1 gene. There were 2 insertions, of 3 and 21 nucleotides, towards the 5′ end of the Newbury2 ORF1 gene at nucleotide positions 130–132 and 157–177. In contrast, compared with the Newbury2 genome, there were 2 insertions, of 9 and 3 nucleotides, in the Jena ORF1 gene, at nucleotide positions 2650–2658 and 2689–2691.

The Newbury2 and Jena ORF1 genes had, overall, 73% nucleotide identity (range 38–83%) (Fig. 1A) and 84% amino acid identity (range 71–92%) (Table 1). The majority of the variation was within the first 500 nucleotides and between nucleotides 2000 and 3000, which included the 3A–B(VPg) region, with only 71%

Table 1. Amino acid identities[a] between regions of the Newbury2 ORF1 polyprotein, ORF2 capsid protein and ORF3 protein compared to bovine, human and murine noroviruses

ORF	Region	Norovirus (Genogroup)				
		Dumfries (III)	Jena (III)	Norwalk (I)	Lordsdale (II)	Murine (Unassigned)
1	Complete	97	84	56	48	41
	N-terminal	95	75	36	31	27
	NTPase	99	92	67	49	42
	3A-3B(VPg)	96	71	40	30	24
	3C-protease	97	88	64	66	49
	3D-polymerase	99	89	73	64	57
2	Complete	97[b]	68[b]	48[b]	43[b]	38
	S-domain	99[b]	86[b]	61[b]	58[b]	54
	P1-domain	97	66	50	46	35
	P2-domain	95	40	22	18	17
3	Complete	96[b]	64[b]	37[b]	28[b]	25

[a]Percent amino acid identities were calculated using GeneDoc (www.psc.edu/biomed/genedoc)

[b]Reported previously [33]

amino acid identity. The NTPase regions of the two viruses showed a remarkably high level of identity (92% amino acid), higher than that of the 3D-polymerase regions. Extensive variation between the Newbury2 and Jena genomes was also seen for the ORF2 and 3 genes, but this was reported previously [30]. All regions of the Newbury2 genome, including the ORF2 and 3 genes reported previously [30], had low levels of amino acid identities with genogroups I and II noroviruses and the murine norovirus (Table 1).

The Newbury2 genome showed moderate nucleotide conservation (11 of 24 nucleotides) between its 5′ terminus region and the region of the ORF1 and 2 overlap (Fig. 1 panel A). This is typical for all members of the family *Caliciviridae*, especially members of the genus *Norovirus* [10] and including the bovine Jena genome. This finding suggests that the transcription/translation mechanism of ORFs 1 and 2 of bovine noroviruses is similar to other caliciviruses. Consistent with Jena, the putative protease cleavage sites, LQGP and LQAP, on either side of the NTPase, plus FQAP and LEGG, on either side of the 3C protease, were conserved for Newbury2 (Fig. 1B). A cleavage site could not be identified for the 3A-B(VPg) region because the conserved human noroviruses dipeptides used to predict the cleavage sites were not present for the bovine noroviruses. The amino acid motifs typical of caliciviruses were present in the Newbury2 ORF1 polyprotein in the NTPase (GPPGIGKT), the 3C protease (GDCG) and the 3D polymerase (GLPSG and YGDD), as previously described for Jena [25].

Phylogenetic analyses of the Newbury2 complete ORF1 protein confirmed that bovine noroviruses formed two distinct clusters in genogroup III, most closely related to, but distinct from, genogroup I (Fig. 2). The internal branch node that

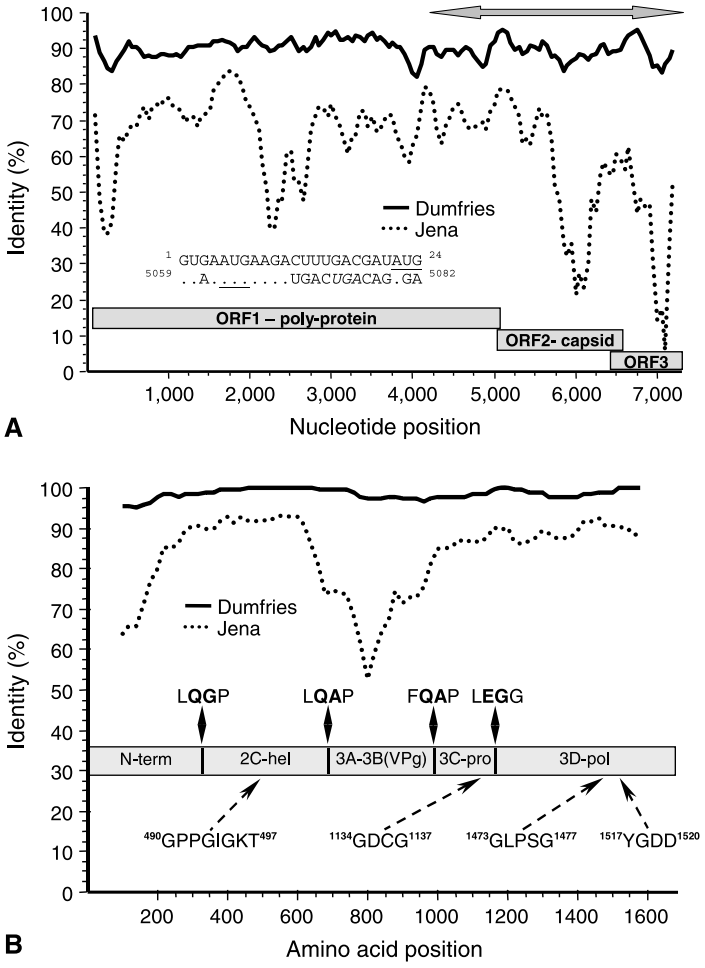

Fig. 1. A. Nucleotide identity plot of the complete genome of Newbury2 compared to those of Jena and Dumfries. The shaded boxes represent the 3 predicted open reading frames (ORFs) for the genotype 2 bovine noroviruses. The shaded arrow above the chart shows the region of the Newbury2 and Dumfries genomes reported previously (33). Comparison of the 24 nucleotides at the 5′ terminus of the Newbury2 genome (nucleotides 1 to 24) with that of the Newbury2 ORF1 and 2 overlap (nucleotides 5059 to 5082) is shown with the initiation codons underlined and the termination codon for ORF1 in italics. **B**. Amino acid identity plot of the complete ORF1 polyprotein of Newbury2 compared to those of Dumfries and Jena. The shaded box shows the likely organisation of the bovine norovirus polyprotein (the term NTPase is used in the text for the 2C-helicase region). The protease cleavage sites above the shaded box were predicted from human noroviruses. The figures either side of the conserved norovirus amino acid motifs, shown below the shaded box, are the locations in the Newbury2 and Dumfries polyproteins and differ slightly from those in the Jena virus because of insertions and deletions in the polyproteins (refer to text)

separated genogroup III from the human genogroups (I and II) was exclusively supported by quartet puzzling statistics or bootstrap values from the parsimony (Protpars) and distance methods (Fitch-Margoliash and UPGMA) of Phylip (data not shown).

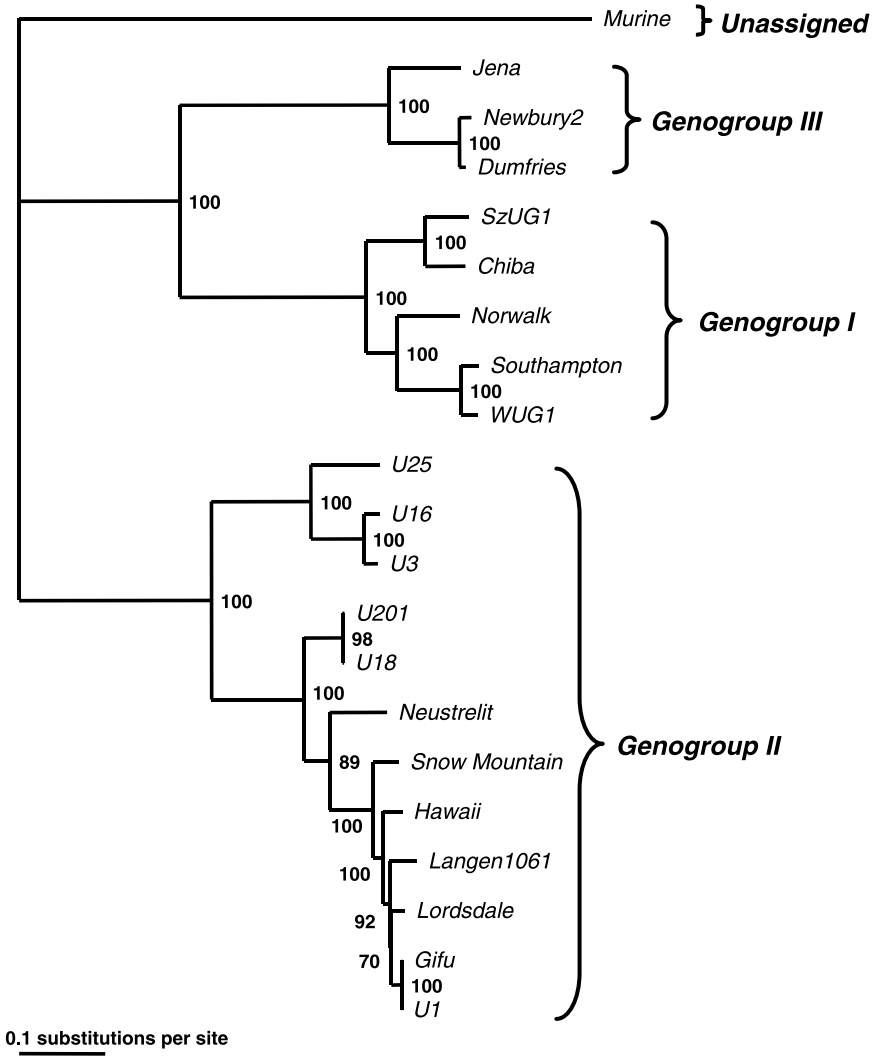

Fig. 2. Phylogenetic analysis by maximum likelihood of the complete ORF1 polyprotein of bovine, human and murine noroviruses using TreePuzzle. Numbers at the internal branch nodes are quartet-puzzling statistics for 1000 replicates. The scale shown for the branch lengths represent substitutions per site

Analysis of the complete Bo/Dumfries/1994/UK genome and ORF1 gene

The complete Dumfries genome was identical to that of Newbury2 in its overall length (7311 nucleotides), organization, and the length of the 3 ORFs. ORF1 was 5055 nucleotides in length, ORF2 was 1569 nucleotides and ORF3 was 849 nucleotides in length. The genome had the same G + C content (57%) as Newbury2. The amino acids motifs in the 2C NTPase, the 3C protease and 3D polymerase, typical of the caliciviruses, were present in the Dumfries genome. The putative protease cleavage sites LQGP and LQAP on either side of the

NTPase, plus FQAP and LEGG on either side of the 3C protease, were also conserved.

The nucleotide and amino acid variation between the Dumfries and Newbury2 ORF1 genes was surprisingly low for two RNA viruses isolated 18 years apart and from disparate geographical locations. Nucleotide identities across the ORF1 gene ranged from 82 to 95% (Fig. 1A). Amino acid identities of the N-terminal, NTPase, 3A–3B (VPg), 3C-protease and 3D-polymerase proteins were high, ranging from 95 to 99% (Table 1). Similar levels of identity were found between the Dumfries and Newbury2 ORF2 and 3 proteins, including the hypervariable capsid P2 region [33]. Because of the high similarity of the Newbury2 and Dumfries genomes, the variation seen between the Dumfries and Jena genomes was almost identical to that between the Newbury2 and Jena genomes. The N-terminal region of Dumfries and Jena had 75% amino acid identity, the NTPase 92%, 3A–3B(VPg) 72%, the 3C-protease 88% and the 3D-polymerase 90% amino acid identity. Extensive variation between the Dumfries and Jena genomes was also seen for the ORF2 and 3 genes (33). Phylogenetic analysis of the complete ORF1 gene (Fig. 2) and the ORF2 capsid and ORF3 genes [33] confirmed that the Dumfries and Newbury2 genomes were closely related but distinct from that of Jena, thus forming two genotypes in genogroup III.

Expression of Bo/Newbury2/1976/UK VLPs and antigenic relationships

Newbury2 VLPs, similar in morphology to native virions, were produced in milligram quantities when the Newbury2 ORF2 capsid gene was expressed using

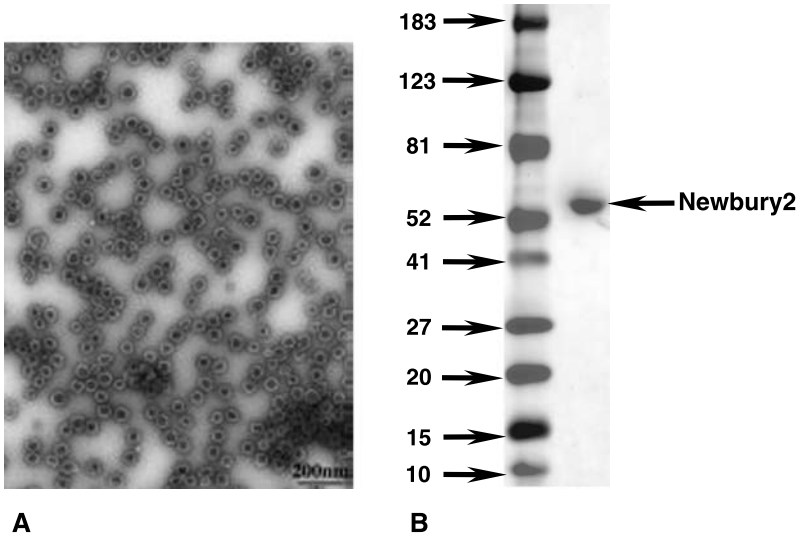

Fig. 3. A. Electron microscopy of CsCl-purified Newbury2 VLPs generated by baculovirus expression of the capsid (ORF2) gene and negatively stained with 2% uranyl acetate. **B**. Protein analysis of CsCl-purified Newbury2 VLPs stained with Ponceau S following SDS-PAGE and western transfer. The arrows with numbers indicate the molecular masses for the marker proteins (Cambrex)

Table 2. Log_{10} serum IgG responses to Newbury2 VLPs in the sera of 4 age-matched gnotobiotic calves which were part of a cross-protection experiment between Newbury2 and Newbury1

Calf[a]	Pre-inoc. IgG titre	Primary inoc.			Primary challenge inoc.			Secondary challenge inoc.		
		Inoculum	Infection index[b]	IgG titre[c]	Inoculum	Infection index[b]	IgG titre[d]	Inoculum	Infection index[b]	IgG titre[e]
P131	<1.7	Newbury2	4	4.1	Newbury2	0	3.9	Newbury2	0	3.7
P137	<1.7	Newbury2	3	4.4	Newbury2	0	4.4	Newbury1	4	3.8
P142	<1.7	Newbury2	3	3.1	Newbury2	0	4.3	Newbury1	5	3.8
Q21	<1.7	None	NR	<1.7	None	NR	<1.7	Newbury2	3	3.5

[a]P131 and Q21 served as challenge controls
[b]Infection indices were previously reported [3]
[c]Serum taken 22 days post inoculation
[d]Serum taken 14 days after the primary challenge inoculation
[e]Serum taken 20 to 21 days after the secondary challenge inoculation

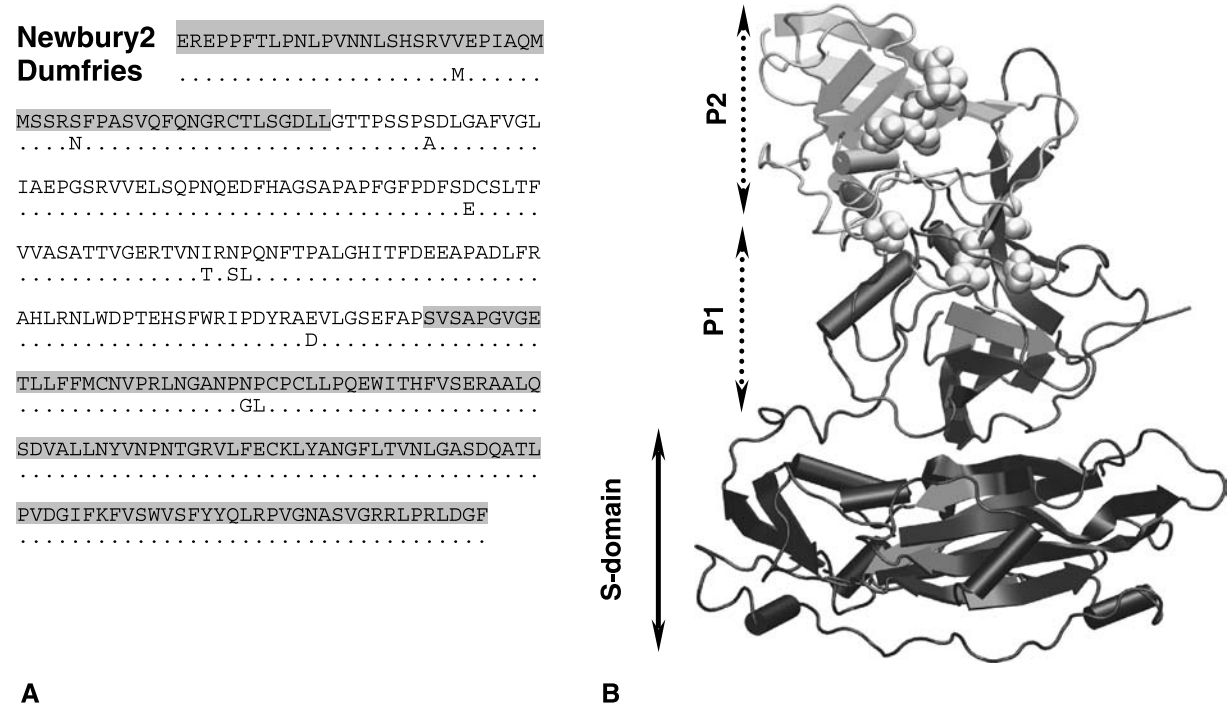

Fig. 4. A. An amino acid alignment of the P domains (amino acids 219–522) of Newbury2 and Dumfries showing the P1 domain (amino acids 219–270 and 397–511; shaded) and the P2 domain (amino acids 271–396 – not shaded) based on Hu/Norwalk/68/US [34]. **B**. A cartoon of a homology model for the S-, P1- and P2-domains of the Newbury2 capsid protein showing the positions of the 10 amino acid substitutions found in the P-domain of the Dumfries capsid protein. The amino acids substituted in the Dumfries capsid protein compared with Newbury2 are represented by space-fill

the baculovirus expression system (Fig. 3A). The molecular mass of the capsid protein monomer from denatured VLPs was approximately 56 kDa by SDS-PAGE (Fig. 3B), in agreement with the molecular mass predicted from translated amino acids of the capsid gene [33]. Denatured Newbury2 VLPs reacted weakly in western blots with a Newbury2 convalescent calf serum but non-denatured Newbury2 VLPs reacted strongly with a high-molecular-weight band (not shown). Good antibody responses were detected by ELISA using the VLPs and sera from four gnotobiotic calves inoculated with Newbury2 virus as a primary or challenge inoculation (mean IgG titre $\log_{10}$ 3.9, range 3.1–4.4) (Table 2). Pre-inoculation sera and convalescent sera from calves infected with the enteric viruses Newbury1, bovine rotavirus UK, bovine coronavirus, bovine astrovirus or the Haden strain of bovine parvovirus did not react ($<\log_{10}$ 1.7). Bo/Newbury2/1976/UK and Bo/Dumfries/1994/UK viruses were indistinguishable in the Newbury2 antibody ELISA. Five sera taken from two calves orally inoculated with the Dumfries virus over a 42-day period had similar titres ($\log_{10}$ 4.0, range 3.7–4.2) to those in the sera from the calves infected with Newbury2. Compared to Newbury2, there were 10 amino acid substitutions throughout the P domain of the capsid protein of Dumfries, 6 in the P2 domain (Fig. 4A). A homology model of the Newbury2 capsid protein showed that the amino acid substitutions in the Dumfries capsid protein formed two clusters in the P-domain, one tight cluster in the P2 domain and a less well-defined cluster in the P1 domain (Fig. 4B). Thus, these two clusters of amino acid substitutions did not prevent the development of cross-reactive antibodies.

Discussion

Complete genomic characterization of the bovine noroviruses, Newbury2 and Dumfries, showed that they had the genomic organization and conserved motifs expected of noroviruses and suggested that Newbury2 was genomically and antigenically representative of genogroup III, genotype 2 bovine noroviruses.

The levels of genomic identity between the Newbury2 and Dumfries ORF1 genes reported in the present study, and between the ORF2 and 3 genes of Newbury2, Dumfries and the Newbury2-like viruses [14, 32, 33, 38, 44, 45], was surprisingly high for RNA viruses identified over almost 3 decades. The tight clustering of the Newbury2 viruses resembled the tight clustering seen, for example, for human genogroup II/4 Lordsdale-type viruses. It remains to be seen whether this tight clustering will persist and whether the number of bovine genotypes will increase as more studies are conducted. The levels of identity observed between the Newbury2 and Dumfries genomes was not a consequence of selection by the RT-PCR primers used to amplify these viruses. Both Newbury2 and Dumfries were originally identified by electron microscopy, from disparate geographical locations and 18 years apart [46; D. Snodgrass, personal communication]. The apparent stability of the genotype 2 bovine norovirus genome will simplify diagnostic tests based on RT-PCR. Most recent studies have used primers designed to the polymerase region that might favour detection of Newbury2-like viruses [33, 38, 44, 45] but the finding that the NTPase was also highly conserved

Table 3. Oligonucleotides used to obtain the nucleotide sequence of the BoCVs Bo/Newbury2/76/UK and Bo/Dumfries/94/UK

Name	Nucleotide sequence (5′ to 3′)	Location in the Newbury2 genome	Location in the Jena genome
JENA01	TGCGATCGCATTGAGAACAAGG	1720–1741	1696–1717
JENA02	AGTCGAAGCCTCCCTGGGGGG	1961–1981	1937–1957
BoCVpro_01	AGATCAGGGAGGAACGTGG	2762–2780	2750–2768
BoCVproR_01	ATCCCGCATCACCTGTTGG	3726–3743	3714–3732
BoCVhel_01	CTGGACCACATGAATCTTGGGCC	1801–1823	1777–1799
BoCVhel_02	TGCGACGGGTTGACTTCCTCG	1828–1849	1805–1825
BoCVhel_03	TTGTTCAAGAAGGACTTCACCC	1921–1942	1897–1918
BoCVhelR_01	ACTGGCCCAAGATTCATGTGGTCC	1803–1826	1779–1802
BoCVhelR_02	TACGAGGAAGTCAACCCGTCG	1831–1851	1807–1827
BoCVhel_04	ATTTGGAATTCTACGAGG	407–424	383–400
BoCVhelR_04	AAGGGTCTTGATGTAGGCC	1302–1320	1278–1296
BoCVpro_02	TCTGCACGATCATCTGGCG	2258–2276	2234–2252
BoCVproR_02	TCCTTCAGGGATGACATGC	3099–2117	3087–3105
BoCV5′RACE_05	ATGTAGCCCTCGTAGAATTCC	431–411	407–387
BoCV5′RACE_06	TGGACGTCGAGGTGGACAGCGC	337–316	281–260
5′JANA01	GTGAATGAAGACTTTGACGATATGG	1–25	1–25

between genotype 1 and 2 bovine noroviruses (92% amino acid identity) offers an opportunity to use RT-PCR primers based on the NTPase region which might identify a wider range of bovine noroviruses, if they exist.

The high amino acid identities in the capsid regions of Newbury2 and Dumfries genomes correlated with the ability of Newbury2 VLPs to detect Dumfries virus antibodies. The results of the present paper, combined with observations that other genotype 2 bovine noroviruses have 94% or greater amino acid identity with Newbury2 [14, 33, 45], suggested that serological assays based on Newbury2 will be suitable to detect genogroup III/2 bovine noroviruses and their antibodies. This view is endorsed by studies with human noroviruses with similar identities in their capsid proteins and which show antigenic cross-reactivity [12, 19]. In contrast, a recent study of the antigenic relationship of Newbury2 and the genotype 1 Jena virus, which have 68% amino acid identify in their capsid proteins, showed that they were poorly related antigenically and that independent ELISAs are required to detect both [31]. Similarly, studies of human norovirus capsid proteins with 73% amino acid identity or less showed little or no cross reactivity in ELISA [12, 19]. Although not tested by ELISA, the capsid amino acid identities of Newbury2 with human genogroup I and II noroviruses of 48 and 43% make it unlikely that the Newbury2 viruses cross-react with human genogroup I and II noroviruses in ELISA. However, it should be noted that a cross-reactive epitope between some genogroup II human noroviruses and the Newbury2 and Jena viruses has been identified using a monoclonal antibody [31].

The lack of reactivity of Newbury1 antiserum in the Newbury2 ELISA reported in the present study, and the lack of cross-protection between the two viruses [3] is explained by the recent finding that Newbury1 belongs to a new genus in the family *Caliciviridae* [30].

The Newbury2 VLPs were composed of a protein of similar molecular mass to VLPs expressed from the Newbury2-like virus Bo/CV186-OH/00/US [14]. They appeared antigenically authentic. Antibody titres in experimental calf sera were similar to those obtained previously [14] and similar to those obtained with human patients [9]. Homologous protection to Newbury2 disease and virus shedding was reported in calves [3], thus allowing calves to be used as an experimental model for immunity to disease. Except for the study by Subekti et al. [42], norovirus disease models using chimpanzees and non-human primates [36, 47] have not produced clinical signs, although serological responses and virus excretion were demonstrated. The calf model allows pre-exposure to norovirus infection to be controlled, but it does have the limitations of production of few offspring and animal size.

Rotaviruses and coronaviruses are recognised as the major viral pathogens in outbreaks of neonatal calf diarrhoea [2, 35, 39]. However, in the UK, up to 30% of calf diarrhoea outbreaks are without an identifiable pathogen [2, 35, 39] and the dam vaccines are said not to be totally effective. The presence of noroviruses might explain some of these undiagnosed outbreaks. The results from the present study will allow the role of bovine noroviruses to be established and vaccines to be developed, if necessary.

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References

1. Ando T, Noel JS, Fankhauser RL (2000) Genetic classification of "Norwalk-like viruses". J Infect Dis 181: S336–S348
2. Andrews AH (2000) Calf enteritis – New information from NADIS. UK VET 5: 30–34
3. Bridger JC, Hall GA, Brown JF (1984) Characterization of a calici-like virus (Newbury agent) found in association with astrovirus in bovine diarrhoea. Infect Immunol 43: 133–138
4. Clarke IN, Lambden PR (2000) Organization and expression of calicivirus genes. J Infect Dis 181: S309–S316
5. Dastjerdi AM, Green J, Gallimore CI, Brown DW, Bridger JC (1999) The bovine Newbury agent-2 is genetically more closely related to human SRSVs than to animal caliciviruses. Virology: 254: 1–5
6. Deng Y, Batten CA, Liu BL, Lambden PR, Elschner M, Gunther H, Otto P, Schnurch P, Eichhorn W, Herbst W, Clarke IN (2003) Studies of epidemiology and seroprevalence of bovine noroviruses in Germany. J Clin Microbiol 41: 2300–2305
7. Dingle KE, Lambden PR, Caul EO, Clarke IN (1995) Human enteric Caliciviridae: the complete genome sequence and expression of virus-like particles from a genetic group II small round structured virus. J Gen Virol 76: 2349–2355

8. Glass PJ, White LJ, Ball JM, Leparc-Goffart I, Hardy ME, Estes MK (2000) Norwalk virus open reading frame 3 encodes a minor structural protein. J Virol 74: 6581–6591
9. Gray JJ, Cunliffe C, Ball J, Graham DY, Desselberge U, Estes MK (1994) Detection of immunoglobulin M (IgM), IgA, and IgG Norwalk virus-specific antibodies by indirect enzyme-linked immunosorbent assay with baculovirus-expressed Norwalk virus capsid antigen in adult volunteers challenged with Norwalk virus. J Clin Microbiol 32: 3059–3063
10. Green KY, Chanock RM, Kapikian AZ (2001) Human caliciviruses. In: Griffin DE, Lamb RA, Martin MA, Roizman B, Straws SE (eds) Fields virology, vol. 1. Lippincott, Williams and Wilkins, Philadelphia, pp 841–874
11. Gunther H, Otto P (1987) Diarrhoea in young calves. 7. Zackenvirus (Jena agent 117/80) – a new diarrhoea pathogen in calves. Arch Exp Veterinarmed 41: 934–938
12. Hale AD, Crawford SE, Ciarlet M, Green J, Gallimore C, Brown DW, Jiang X, Estes MK (1999) Expression and self-assembly of Grimsby virus: antigenic distinction from Norwalk and Mexico viruses. Clin Diagn Lab Immunol 6: 142–145
13. Han MG, Smiley JR, Thomas C, Saif LJ (2004) Genetic recombination between two genotypes of genogroup III bovine noroviruses (BoNVs) and capsid sequence diversity among BoNVs and Nebraska-like bovine enteric caliciviruses. J Clin Microbiol 42: 5214–5224
14. Han MG, Wang Q, Smiley JR, Chang KO, Saif LJ (2005) Self-assembly of the recombinant capsid protein of a bovine norovirus (BoNV) into virus-like particles and evaluation of cross-reactivity of BoNV with human noroviruses. J Clin Microbiol 43: 778–785
15. Humphrey W, Dalke A, Schulten K (1996) VMD: visual molecular dynamics 1. J Mol Graph 14: 33–38
16. Jiang X, Graham DY, Wang KN, Estes MK (1990) Norwalk virus genome cloning and characterization. Science 250: 1580–1583
17. Jiang X, Wang M, Graham DY, Estes MK (1992) Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein. J Virol 66: 6527–6532
18. Jiang X, Cubitt D, Hu J, Dai X, Treanor J, Matson DO, Pickering LK (1995) Development of an ELISA to detect MX virus, a human calicivirus in the snow Mountain agent genogroup. J Gen Virol 76: 2739–2747
19. Jiang X, Zhong WM, Farkas T, Huang PW, Wilton N, Barrett E, Fulton D, Morrow R, Matson DO (2002) Baculovirus expression and antigenic characterization of the capsid proteins of three Norwalk-like viruses. Arch Virol 147: 119–130
20. Karst SM, Wobus CE, Lay M, Davidson J, Virgin HW (2003) STAT1-dependent innate immunity to a Norwalk-like virus. Science 299: 1575–1578
21. Katayama K, Shirato-Horikoshi H, Kojima S, Kageyama T, Oka T, Hoshino F, Fukushi S, Shinohara M, Uchida K, Suzuki Y, Gojobori T, Takeda N (2002) Phylogenetic analysis of the complete genome of 18 Norwalk-like viruses. Virology 299: 225–239
22. Labbe M, Charpilienne A, Crawford SE, Estes MK, Cohen J (1991) Expression of rotavirus VP2 produces empty corelike particles. J Virol 65: 2946–2952
23. Lambden PR, Caul EO, Ashley CR, Clarke IN (1993) Sequence and genome organization of a human small round-structured (Norwalk-like) virus. Science 259: 516–519
24. Lindesmith L, Moe C, Lependu J, Frelinger JA, Treanor J, Baric RS (2005) Cellular and humoral immunity following Snow Mountain virus challenge. J Virol 79: 2900–2909
25. Liu BL, Lambden PR, Gunther H, Otto P, Elschner M, Clarke IN (1999) Molecular characterization of a bovine enteric calicivirus: relationship to the Norwalk-like viruses. J Virol 73: 819–825
26. Lochridge VP, Hardy ME (2003) Snow Mountain virus genome sequence and virus-like particle assembly. Virus Genes 26: 71–82

27. Lole KS, Bollinger RC, Paranjape RS, Gadkari D, Kulkarni SS, Novak NG, Ingersoll R, Sheppard HW, Ray SC (1999) Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. J Virol 73: 152–160
28. Mendez II, Hermann LL, Hazelton PR, Coombs KM (2000) A comparative analysis of freon substitutes in the purification of reovirus and calicivirus. J Virol Methods 90: 59–67
29. Noel JS, Ando T, Leite JP, Green KY, Dingle KE, Estes MK, Seto Y, Monroe SS, Glass RI (1997) Correlation of patient immune responses with genetically characterized small round-structured viruses involved in outbreaks of nonbacterial acute gastroenteritis in the United States, 1990 to 1995. J Med Virol 53: 372–383
30. Oliver SL, Asobayire E, Dastjerdi AM, Bridger JC (2006) Genomic characterization of the unclassified bovine enteric virus Newbury agent-1 (Newbury1) endorses a new genus in the family *Caliciviridae*. Virology 350: 240–250
31. Oliver SL, Batten C, Deng Y, Elschner M, Otto P, Charpilienne A, Clarke IN, Bridger JC, Lambden P (2006) Genotype-1 and genotype-2 bovine noroviruses are antigenically distinct but share a cross-reactive epitope with human noroviruses. J Clin Microbiol 44: 992–998
32. Oliver SL, Brown DW, Green J, Bridger JC (2004) A chimeric bovine enteric calicivirus: evidence for genomic recombination in genogroup III of the Norovirus genus of the *Caliciviridae*. Virology 326: 231–239
33. Oliver SL, Dastjerdi AM, Wong S, El Attar L, Gallimore C, Brown DW, Green J, Bridger JC (2003) Molecular characterization of bovine enteric caliciviruses: a distinct third genogroup of noroviruses (Norwalk-like viruses) unlikely to be of risk to humans. J Virol 77: 2789–2798
34. Prasad BV, Hardy ME, Dokland T, Bella J, Rossmann MG, Estes MK (1999) X-ray crystallographic structure of the Norwalk virus capsid. Science 286: 287–290
35. Reynolds DJ, Morgan JH, Chanter N, Jones PW, Bridger JC, Debney TG, Bunch KJ (1986) Microbiology of calf diarrhoea in southern Britain. Vet Rec 119: 34–39
36. Rockx BH, Bogers WM, Heeney JL, van Amerongen G, Koopmans MP (2005) Experimental norovirus infections in non-human primates. J Med Virol 75: 313–320
37. Schmidt HA, Strimmer K, Vingron M, von Haeseler A (2002) TREE-PUZZLE: maximum likelihood phylogenetic analysis using quartets and parallel computing. Bioinformatics 18: 502–504
38. Smiley JR, Hoet AE, Traven M, Tsunemitsu H, Saif LJ (2003) Reverse transcription-PCR assays for detection of bovine enteric caliciviruses (BEC) and analysis of the genetic relationships among BEC and human caliciviruses. J Clin Microbiol 41: 3089–3099
39. Snodgrass DR, Terzolo HR, Sherwood D, Campbell I, Menzies JD, Synge BA (1986) Aetiology of diarrhoea in young calves. Vet Rec 119: 31–34
40. Someya Y, Takeda N, Miyamura T (2000) Complete nucleotide sequence of the Chiba virus genome and functional expression of the 3C-like protease in *Escherichia coli*. Virology 278: 490–500
41. Staden R, Beal KF, Bonfield JK (2000) The Staden package (1998) Methods Mol Biol 132: 115–130
42. Subekti DS, Tjaniadi P, Lesmana M, McArdle J, Iskandriati D, Budiarsa IN, Walujo P, Suparto IH, Winoto I, Campbell JR, Porter KR, Sajuthi D, Ansari AA, Oyofo BA (2002) Experimental infection of Macaca nemestrina with a Toronto Norwalk-like virus of epidemic viral gastroenteritis. J Med Virol 66: 400–406
43. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25: 4876–4882

44. van der Poel WH, van der Heide R, Verschoor F, Gelderblom H, Vinje J, Koopmans MP (2003) Epidemiology of Norwalk-like virus infections in cattle in The Netherlands. Vet Microbiol 92: 297–309
45. Wise AG, Monroe SS, Hanson LE, Grooms DL, Sockett D, Maes RK (2004) Molecular characterization of noroviruses detected in diarrheic stools of Michigan and Wisconsin dairy calves: circulation of two distinct subgroups. Virus Res 100: 165–177
46. Woode GN, Bridger JC (1978) Isolation of small viruses resembling astroviruses and caliciviruses from acute enteritis of calves. J Med Microbiol 11: 441–452
47. Wyatt RG, Greenberg HB, Dalgard DW, Allen WP, Sly DL Thornhill TS, Chanock RM, Kapikian AZ (1978) Experimental infection of chimpanzees with the Norwalk agent of epidemic viral gastroenteritis. J Med Virol 2: 89–96

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